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Saccharomyces cerevisiae and *Kluyveromyces marxianus* Cocultures Allow Reduction of Fermentable Oligo-, Di-, and Monosaccharides and Polyols Levels in Whole Wheat Bread

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Supporting Information

ABSTRACT: Fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) are small molecules that are poorly absorbed in the small intestine and rapidly fermented in the large intestine. There is evidence that a diet low in FODMAPs reduces abdominal symptoms in approximately 70% of the patients suffering from irritable bowel syndrome. Wheat contains relatively high fructan levels and is therefore a major source of FODMAPs in our diet. In this study, a yeast-based strategy was developed to reduce FODMAP levels in (whole wheat) bread. Fermentation of dough with an inulinase-secreting *Kluyveromyces marxianus* strain allowed to reduce fructan levels in the final product by more than 90%, while only 56% reduction was achieved when a control *Saccharomyces cerevisiae* strain was used. To ensure sufficient CO_2 production, cocultures of *S. cerevisiae* and *K. marxianus* were prepared. Bread prepared with a coculture of *K. marxianus* and *S. cerevisiae* had fructan levels ≤0.2% dm, and a loaf volume comparable with that of control bread. Therefore, this approach is suitable to effectively reduce FODMAP levels in bread.

KEYWORDS: *Saccharomyces cerevisiae, Kluyveromyces marxianus, wheat, fermentation, FODMAPs, invertase, inulinase*

INTRODUCTION

Fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) are small, osmotically active molecules that are poorly absorbed in the small intestine and rapidly fermented by bacteria present in the large intestine. Fructose (in excess of glucose), lactose, fructo- and galacto-oligosaccharides, and polyols all belong to the FODMAP group.[1] In contrast to fructo-oligosaccharides and galacto-oligosaccharides, which are never absorbed in the small intestine, fructose is conditionally nondigested in the small intestine (e.g., fructose malabsorption).[2,3] Indeed, the capacity to absorb fructose has been shown to vary substantially between individuals.[2] Glucose is not a FODMAP, but its presence in food products enhances the absorption of fructose in the small intestine and hence reduces the induction of symptoms associated with fructose malabsorption. At equal or greater concentrations of glucose, fructose will be largely and rapidly absorbed via GLUT2, a very efficient fructose and glucose facilitative transporter. When fructose is present in excess of glucose, however, its uptake is dependent on the slower GLUT5 pathway.[4]

There is evidence that the intake of FODMAPs induces abdominal symptoms such as pain, bloating, nausea, and disturbed bowel habit in people suffering from irritable bowel syndrome (IBS).[5−9] IBS is a chronic functional gastrointestinal disorder with a prevalence up to 12% in the European population and between 7 and 15% worldwide.[10,11] Several studies showed that a diet low in FODMAPs alleviates symptoms in approximately 70% of IBS patients.[11−17] Furthermore, the uptake of FODMAPs was suggested to be associated with the occurrence of gastro-intestinal complaints in people suffering from Crohn's disease and nonceliac gluten sensitivity (NCGS).[18−20]

Patients suffering from IBS are advised to follow a diet that is limited in FODMAPs.[1] This implies that they should avoid the consumption of wheat-derived products like bread, as wheat grains contain relatively high fructan levels (0.9−2.7% dm).[21] Fructans are linear or branched carbohydrates consisting mainly or exclusively of fructose units and maximally one glucose unit per molecule.[22] The small intestine lacks hydrolases capable of breaking fructose-fructose bonds and fructans cannot be transported across the epithelium, which explains their limited absorption in the small intestine. A previous study showed that 87% of the ingested fructans (inulin) could be recovered from the small intestinal output in ileostomy subjects.[23]

Fructans are not only relevant from a nutritional point of view but also because of their role as an energy source for yeast during dough fermentation. Indeed, fructans are partially degraded to glucose and fructose during dough fermentation. The enzyme responsible for fructan hydrolysis is invertase, which is produced by *Saccharomyces cerevisiae* (*S. cerevisiae*) yeast cells during fermentation.[24,25] Depending on the time of fermentation and the yeast dosage, between 50 and 80% of the fructans initially present in flour or whole meal are degraded

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during bread making.[25−27] Despite this degradation, fructan levels in wheat and rye bread products are nearly always above the cutoff value that is considered at-risk for inducing symptoms in people that are sensitive to FODMAPs (>0.2 g per serve).[28] The complete degradation of wheat grain fructans during bread making with yeast as leavening agent has never been described.

Although they contain FODMAPs, wheat based products are nutritious staple foods and avoiding their consumption may result in nutritional deficiencies. Therefore, adaptations of the bread making process that allow a complete degradation of fructan in bread are desirable. A recent study of Ziegler et al.[29] revealed that prolonged proofing times (>4 h) allow one to diminish FODMAP levels from wheat flour to bread by 90%.[29] Prolonged proofing times are, however, often not desirable from an economical point of view.

In this study, a yeast-based strategy to reduce fructan levels in whole meal bread was developed. As baker's yeast invertase seems insufficient to degrade fructans completely, an alternative yeast species that secretes inulinase, an efficient fructan degrading enzyme, was selected. *Kluyveromyces marxianus* (*K. marxianus*) is phylogenetically related to *S. cerevisiae*.[30] The capacity of *K. marxianus* to assimilate sugars like lactose and inulin and its extremely high growth rate are desirable traits for biotechnology applications.[31] Its long history of safe use in food products facilitated its Generally Regarded As Safe (GRAS) and Qualified Presumption of Safety (QPS) ratification in the United States of America and European Union, respectively.[30]

Inulinase and invertase differ in their specificity toward higher-molecular-weight fructans of the inulin-type. The S/I ratio (relative activities toward sucrose and inulin) is higher for invertase than for inulinase, which is related to the low specificity of invertase toward fructose polymers with a high degree of polymerization (DP) like inulin.[32] Inulinase produced by *K. marxianus* has both a cell wall associated form and a secreted form, while invertase produced by *S. cerevisiae* is always retained in the cell wall and not secreted into the dough.[33] This might imply that the fructans present in dough are more accessible for *K. marxianus* inulinase than for *S. cerevisiae* invertase. For these reasons, wheat grain fructans might be degraded to a greater extent by inulinase from *K. marxianus* than by invertase from *S. cerevisiae*.

The goal of this study was therefore to examine whether the use of *K. marxianus* strains as leavening agents can enhance the degradation of wheat grain fructans during fermentation and hence can reduce FODMAP levels in (whole meal) bread. To that end, whole meal dough samples were prepared with (i) a commercial *S. cerevisiae* bakery strain, (ii) a *K. marxianus* strain, and (iii) cocultures of both. During dough fermentation, the CO_2 production rate and FODMAP levels were analyzed. Dough samples were baked and bread volume and FODMAP content of the breads were measured. The results of this study might lead to a yeast-based strategy to reduce FODMAP levels in (whole meal) dough and bread, which is relevant for patients suffering from IBS.

It should be noted that the potential positive health effect of breads with reduced FODMAP levels, and more specifically fructan levels, should not be generalized to the whole population. As fructan is a dietary fiber,[34] the intake of whole meal breads that are rich in fructans, but also in other dietary fibers like, for example, cellulose and arabinoxylan, is beneficial for people with a healthy gastrointestinal tract.[35] Reduction of fructan levels in cereal bread products is hence only valuable for people suffering from gastrointestinal disorders like IBS but not for healthy people that could actually benefit from the uptake of fructans.

MATERIALS AND METHODS

Materials. Wheat variety Terroir was obtained from the experimental site of the Université de Liege (Agro-bio Tech, Gembloux, Belgium). Terroir wheat was milled into flour with a Buhler MLU-202 laboratory mill, with a milling yield of 69.3%. The bran and shorts fractions were further reduced in size (<500 μm) with a Cyclotec 1093 sample mill (FOSS, Höganäs, Sweden), after which they were added to the flour fraction in their original proportions to produce whole meal. Terroir whole meal was finally enriched with 5% vital wheat gluten with a protein content of 82.4% (w/w) (Tereos Syral, Aalst, Belgium).

A fructanase mixture (E-FRMXLQ) containing exoinulinase (2000 U/mL on kestose at 40 °C) and endoinulinase [100 U/mL on inulin (Orafti HP) at 40 °C] was obtained from Megazyme (Bray, Ireland). Invertase (I4504) from *S. cerevisiae* (≥300 U/mg solid on sucrose at 55 °C) was obtained from Sigma-Aldrich (Bornem, Belgium). A commercial *S. cerevisae* bakery strain (Y243) and three different *K. marxianus* strains (NCYC587, MUCL29917, and MUCL53775) were obtained from the collection of the VIB Laboratory for Systems Biology (KU Leuven, Belgium). *S. cerevisiae* Y243 is referred to as Sc1 in this paper, and *K. marxianus* strains NCYC587, MUCL29917, and MUCL53775 are referred to as Km1, Km2, and Km3, respectively. The three *K. marxianus* strains are known to secrete inulinase.

Yeast extract and balanced peptone were provided by Lab M (Brussels, Belgium). All other chemicals and reagents were purchased from Sigma-Aldrich and were of analytical grade.

Whole Meal Characterization. The protein content (N x 5.7) of Terroir whole meal was determined using an automated Dumas protein analysis system (EAS, VarioMax N/CN, Elt, Gouda, The Netherlands) following an adapted version of AOAC method 990.03.[36] Terroir whole meal had a protein content of 11.4% on dry matter (dm) base. The damaged starch content of Terroir whole meal (7.89 ± 0.23% dm) was determined using a colorimetric assay (Megazyme) based on AACCI method 76-31.[37] The falling number of Terroir whole meal (297 ± 2 s) was determined according to AACCI method 56-81.03.[37] Optimal baking absorption and mixing time were determined using Farinograph (Brabender, Duisburg, Germany) and Mixograph (National Manufacturing, Lincoln, NE) analyses according to AACCI Methods 54-21.02 and 54-40.02, respectively.[37] All measurements were carried out in triplicate.

Preparation and Growth of Yeast Cells. Yeast precultures, made by suspending a yeast colony in 5 mL of YPD (1.0% w/v yeast extract, 2.0% w/v balanced peptone, and 2.0% w/v glucose) were shaken (250 rpm) overnight at 30 °C. After 16 h, 3 mL of the preculture was used to inoculate 300 mL of YPD in a baffled Erlenmeyer flask. This second culture was shaken (250 rpm) overnight at 30 °C. The next morning, the optical density (OD) at 595 nm was measured with a microplate reader (Bio-Rad Laboratories, Nazareth, Belgium). For dough preparation, the yeast cells were harvested at an OD of 1.1−1.2 (*S. cerevisiae*) and 1.2−1.3 (*K. marxianus*) by centrifugation (3 min, 870*g*) using a benchtop centrifuge (model EBA 21, Hettich Lab Technology, Massachusetts). Preliminary experiments showed that harvesting the cells at these OD values resulted in maximal fermentation rates in dough. Cells were washed with sterile dH_2O before inoculation in dough. Experiments were always performed with three biological replicates.

The growth profiles of the *S. cerevisiae* bakery strain and the *K. marxianus* strains were determined with the Bioscreen C (Thermo Fisher Scientific, Aalst, Belgium) during 96 h of incubation at 30 °C with continuous shaking. The OD was measured every 15 min.

Dough and Bread Making. Dough was prepared in triplicate using the straight dough method described by Shogren & Finney,[38] using the following formula: 10.0 g of whole meal (on a 14% moisture basis), 1.5% (w/w) sodium chloride, and 5.3% (w/w) freshly harvested yeast, unless specified otherwise. The moisture content of

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Table 1. Composition of the Different Yeast Cultures That Were Used in This Study

yeast culture	yeast strain code	composition	yeast dosage (g yeast pellet/100 g flour) (%)	yeast dosage (g yeast dry matter/100 g flour) (%)
monoculture Sc1	Y243	*Saccharomyces cerevisiae* Sc1	5.30	1.06
monoculture Km1	NCYC587	*Kluyveromyces marxianus* Km1	5.30	1.06
coculture (single dosage)	Y243	*Saccharomyces cerevisiae* Sc1	2.65	0.53
	NCYC587	*Kluyveromyces marxianus* Km1	2.65	0.53
coculture (double dosage)	Y243	*Saccharomyces cerevisiae* Sc1	5.30	1.06
	NCYC587	*Kluyveromyces marxianus* Km1	5.30	1.06

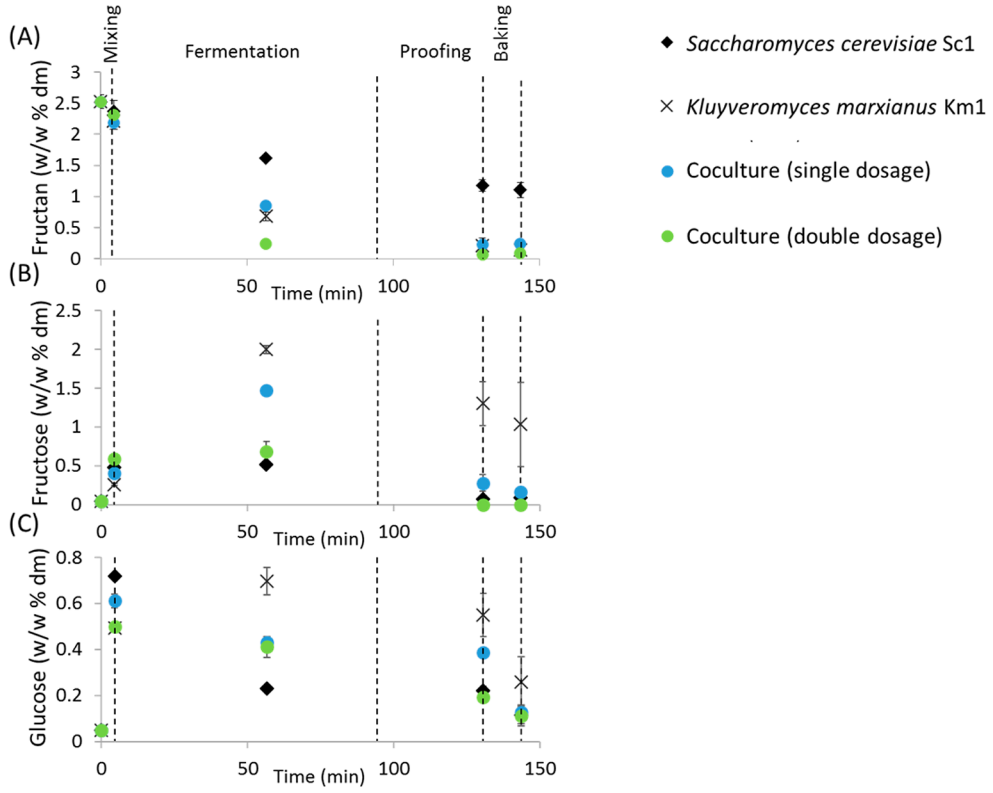

Figure 1. Fructan (A), fructose (B), and glucose (C) concentrations in dough and bread samples fermented with the *Saccharomyces cerevisiae* bakery strain Sc1 (black rhombus), *Kluyveromyces marxianus* strain Km1 (gray cross), a single dosage coculture (blue circle), or a double dosage coculture (green circle). The single dosage coculture contains 1.06% yeast dry matter (on whole meal basis), and the double dosage contains 2.12% yeast dry matter (on whole meal basis). Fructan/fructose/glucose concentrations are expressed as weight percentages on whole meal dry matter base (w/w % dm). The first time point (t = 0) shows fructan/fructose/glucose concentrations in Terroir whole meal. The last time point represents fructan/fructose/glucose concentrations in bread after baking. Vertical dashed lines separate the four consecutive steps of bread making: mixing, fermentation, proofing, and baking. Error bars are standard deviations on measurements of biological triplicates.

the freshly harvested yeast pellets was approximately 80−85% (compared with 65−70% for commercial fresh block yeast). The ingredients were mixed in a 10 g pin bowl mixer (National Manufacturing) for 4 min 30 s. Fermentation and proofing were performed in a fermentation cabinet (National Manufacturing) at 30 °C and 90% relative humidity for 90 and 36 min, respectively. Fermenting doughs were punched at 52, 77, and 90 min of fermentation. Dough samples were taken after mixing (4.5 min), after the first punch (56.5 min), and after proofing (130.5 min). The dough samples were frozen in liquid nitrogen, lyophilized, and ground with a laboratory mill to a powder prior to saccharide analysis. After proofing, doughs were baked for 13 min at 232 °C in a rotary oven (National Manufacturing). Breads were subsequently cooled for 2 h, and their volume was determined with a Volscan profiler (Stable Micro Systems, Godalming, Surrey, U.K.). After the volume measurement, breads were frozen in liquid nitrogen, lyophilized, and ground with a laboratory mill to a powder prior to saccharide analysis.

Gas Production Measurement. The volume of gas produced in dough as a function of fermentation time was measured using a Risograph instrument (National Manufacturing). Doughs were prepared as described earlier and were left to ferment for 300 min at 30 °C in the Risograph chambers. Gas production was measured every minute.

Quantification of Mono-, Di-, and Trisaccharides. Mono-, di-, and trisaccharides were extracted with hot water and subsequently quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as previously described by Struyf et al.[39] Saccharide concentrations were expressed as weight percentages on whole meal dry matter base (% dm). The saccharides present in Terroir whole meal were previously quantified and described.[39] Glucose, fructose, and maltose concentrations in

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Terroir whole meal were ≤0.1% dm for every saccharide. Sucrose and raffinose concentrations in Terroir whole meal were 0.94 ± 0.04% dm and 0.29 ± 0.04% dm, respectively.

Quantification of Fructan. Quantification of fructan in the lyophilized dough and bread powders was performed with HPAEC-PAD after mild acid hydrolysis, as described by Verspreet et al.[40] Saccharide concentrations were expressed as weight percentages on whole meal dry matter base (% dm). The fructan content in Terroir whole meal was 2.52 ± 0.11% dm.

Incubation of Different Substrates with Fructan-Degrading Enzymes. Three different substrates [fructo-oligosaccharides (FOS), inulin, and wheat grain fructans] were incubated with invertase (I4504) and inulinase (E-FRMXLQ) to assess the specificity of these enzymes toward substrates with a different DP. FOS (Orafti P95, Beneo) had a DP range of 2 to 8. Inulin HP (Orafti HP, Beneo) had an average DP ≥ 23. Enzymes and substrates were dissolved in sodium acetate buffer (50 mM, pH 5). Wheat grain fructans were extracted from whole meal (variety Atomic) and had a mean DP of 5.7. An aliquot of 150 μL of FOS or inulin solution (0.5 mg/mL) was incubated with 100 U of enzyme solution [invertase (70 μL) or inulinase (50 μL)] for 2 h at 50 °C. After incubation, the samples were boiled for 10 min to inactivate the enzymes. Wheat grain fructans were extracted from whole meal by addition of 15 mL boiling water and subsequently 1 h of shaking in a water bath at 80 °C. After extraction, samples were centrifuged and an aliquot of 150 μL of the supernatant was incubated with 100 U of enzyme solution [invertase (70 μL) or inulinase (50 μL)] for 2 h at 50 °C. After incubation, the samples were boiled for 10 min to inactivate the enzymes. Fructan levels were determined as described above and experiments were always performed in triplicate.

Statistical Analysis. Dough or bread samples were prepared in triplicate and saccharide concentrations were quantified once in each dough or bread sample, unless specified otherwise. The data were analyzed using statistical software JMP Pro 12. Significant differences were determined by one-way analysis of variance using JMP Pro software 12 (SAS Institute, Cary, NC), with comparison of mean values using the Tukey test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Analysis of FODMAP Levels in Dough and Bread Samples Fermented with Different Yeast Cultures. Fructan degradation was evaluated during the bread making procedure of Shogren and Finney.[38] The different yeast cultures that were used as leavening agents, represented in Table 1, include *S. cerevisiae* and *K. marxianus* monocultures but also cocultures of both species. The use of suspended cocultures of *K. marxianus* and *S. cerevisiae* strains has been described earlier in the scientific literature, for example, in the context of ethanol fermentation.[41]

Figure 1A shows fructan levels in dough and bread samples measured after mixing (4.5 min), after the first punch (56.5 min), after proofing (130.5 min), and after baking (143.5 min). *S. cerevisiae* bakery strain Sc1 degraded the fructan present in whole meal slower and to a lower extent than *K. marxianus* strain Km1 and the cocultures.

After mixing, only minor differences were detected in fructan levels between doughs fermented with the different yeast cultures. After the first punch, however, fructan levels present in the different dough samples were ranked as follows: Sc1 (1.6% dm) > single dosage coculture (0.8% dm) > Km1 (0.7% dm) > double dosage coculture (0.2% dm). Dough samples prepared with the double dosage coculture contained a higher amount of yeast, which means that more fructan degrading enzymes (invertase and inulinase) were produced, explaining the fast degradation of fructan in these dough samples.

After proofing, the fructan level in dough fermented with Sc1 was still above 1% dm (1.2% dm). In dough fermented with the Km1 monoculture, the single dosage coculture and the double dosage coculture, fructan levels were, respectively, 0.2% dm, 0.2% dm, and <0.1% dm. As described previously by Verspreet et al.,[25] fructan levels were not significantly reduced during the baking phase of bread making. This means that breads prepared with Sc1 contained >1% dm fructan, while fructan levels in breads prepared with Km1, the single dosage coculture and the double dosage coculture were 0.2% dm, 0.2% dm, and <0.1% dm, respectively. It has been described that foods containing >0.2 g of fructans in an average serving quantity of the food are considered at-risk for inducing symptoms in IBS subjects.[1,3] On the basis of the proposed threshold value (0.2 g per serving, one serving is 50 g of bread), fructan levels in breads prepared with Km1 and the cocultures were low enough to minimize symptom induction in FODMAP sensitive people.

More than 90% of the fructans initially present in whole meal were degraded by Km1 and the cocultures, while only 56% was degraded when a monoculture of Sc1 was used. Inulinase thus seems much more effective in degrading wheat grain fructans during bread making than invertase, which might be explained by the different substrate specificity of both enzymes.[32] This will be discussed further in the last paragraph.

Next to fructan, also fructose levels in doughs and breads prepared with the different cultures were analyzed, since fructose is also a FODMAP for people that suffer from fructose malabsorption.[1,3] Fructose levels in dough and bread samples prepared with the different cultures, measured at different time points during the bread making process, are represented in Figure 1B. Due the fast degradation of fructan, fructose levels increased during mixing and the first hour of fermentation.[39] Indeed, relatively high fructose levels were present in all dough samples after the first punching step. Fructose concentrations were the highest in dough fermented with the Km1 monoculture, which might indicate that this culture fermented to a lower extent than the other cultures. Indeed, it might be possible that Km1 consumed the hydrolysis products of fructan more slowly than the other cultures. Fructose concentrations were the lowest in doughs fermented with the Sc1 monoculture and the double dosage coculture. The latter might be explained by the high fermentation rate of this culture and hence the fast consumption of sugars, as a relatively high dosage of yeast was present in this coculture. The low fructose concentration in dough fermented with the Sc1, on the other hand, might be related to the lower fructan degrading capacity of this strain. In all cases, except for breads prepared with the Km1 monoculture, fructose levels were ≤0.2% dm. Foods and beverages containing >0.5 g of fructose in excess of glucose per 100 g are considered at-risk for inducing symptoms for people with fructose malabsorption.[1,3] This implies that breads prepared with the Km1 monoculture might still induce symptoms in people with fructose malabsorption. Fructose levels of breads prepared with the cocultures were below 0.5 g/100 g.

Figure 1C represents glucose levels in dough and bread samples prepared with the different cultures at different time points during the bread making process. As mentioned previously, glucose is not a FODMAP, but its presence in food products can enhance the absorption of fructose. Glucose levels in doughs fermented with Km1 and the cocultures were always lower than fructose levels (Figure 1B,C), indicating that fructose was present in excess. This observations might be

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explained by the fact that (i) less glucose is released during fermentation compared with fructose as fructan mainly consists of fructose units and/or (ii) glucose is the preferred sugar source of yeast over fructose.[42] After baking, however, both glucose and fructose levels in the breads were very low (≤0.3% dm) except for breads prepared with the Km1 monoculture. Breads prepared with Km1 contained fructose levels (±1% dm) that were clearly higher than glucose levels (±0.3% dm), which means that fructose was present in excess and that IBS symptoms might be induced after consumption of these breads.

These results indicate that doughs and breads prepared with both cocultures contained FODMAP (fructose and fructan) levels that were under the cutoff value that is considered at-risk for inducing symptoms in people sensitive to FODMAPs. Since the double dosage coculture contains a relatively high amount of yeast cells, which might negatively affect dough rheology and bread taste, the single dosage coculture is probably more suitable for bread dough fermentations.

Comparison of CO_2 Production Rate of Different Yeast Cultures during Dough Fermentation. In order to prepare qualitative breads with the different yeast cultures, their CO_2 production rate in dough should be sufficiently high. Therefore, the CO_2 production rate (mL of CO_2/min) of the *S. cerevisiae* bakery strain Sc1, *K. marxianus* strain Km1, and cocultures of both strains was analyzed during Terroir whole meal dough fermentation (Figure 2).

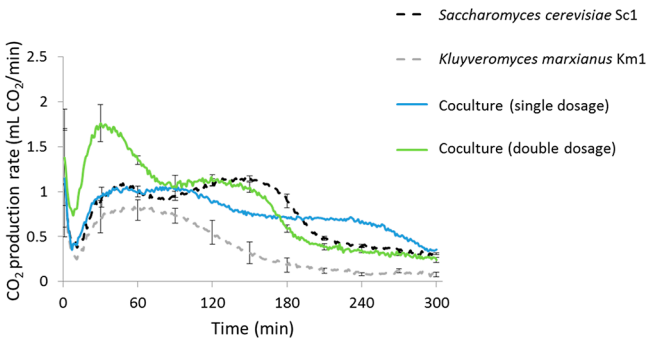

Figure 2. CO_2 production rate (mL of CO_2/min) of the *Saccharomyces cerevisiae* bakery strain Sc1 (black, broken line), *Kluyveromyces marxianus* strain Km1 (gray, broken line), a single dosage coculture (blue, full line) and a double dosage coculture (green, full line) during Terroir whole meal dough fermentation (30 °C). The single dosage coculture contains 1.06% yeast dry matter (on whole meal basis), the double dosage contains 2.12% yeast dry matter (on whole meal basis). Vertical bars represent standard deviations on measurements of biological triplicates.

The CO_2 production rate of Sc1 reached 1.0 mL/min after 30 min of fermentation. After approximately 70 min of fermentation, a slight drop in CO_2 production rate was observed, caused by the adaptation of the yeast cells to the consumption of maltose.[39] Indeed, during the first hour of fermentation, yeast cells mainly consume glucose and fructose, which are the hydrolysis products of fructan and sucrose. When these are depleted, yeast cells shift to the consumption of maltose. After the drop, the CO_2 production rate returned back to its maximal rate and only dropped to lower levels after approximately 180 min of fermentation due to depletion of maltose.

The CO_2 production rate of Km1 in Terroir whole meal dough was lower than that of Sc1. After approximately 30 min of fermentation, a maximal CO_2 production rate of 0.7−0.8 mL/min was reached. This lower CO_2 production rate is correlated with the lower rate of fructose and glucose consumption by Km1 compared with Sc1, as shown in Figure 1B,C. The CO_2 production rate in dough fermented with Km1 already dropped to levels <0.5 mL/min after 120 min of fermentation. This early drop in CO_2 production is related to the fact that *K. marxianus* strains are unable to ferment maltose.[43,44] The consumption of maltose is determinative for the total length of productive fermentation period, and a lack of maltose consumption therefore shortens the total productive fermentation time.[39,45]

As the CO_2 production rate of Km1 was relatively low and dropped quickly, cocultures containing both Sc1 and Km1 seem necessary for the production of breads with a sufficiently high loaf volume. The CO_2 production rate (mL of CO_2/min) of the cocultures in Terroir whole meal dough is represented in Figure 2. The CO_2 production rate of the single dosage coculture in Terroir whole meal dough was similar to the CO_2 production rate of the Sc1 monoculture during the first 2 h of fermentation. This indicates that all cells present in the culture were consuming glucose and fructose, derived from sucrose and fructan hydrolysis. After 2 h of fermentation, the CO_2 production rate of the single dosage coculture dropped slightly to 0.7−0.8 mL/min, while the CO_2 production rate of the Sc1 monoculture remained high (1.1 mL/min). This drop is probably related to the fact that fructose and glucose were nearly depleted after 2 h of fermentation (Figure 1B,C) and the cells need to shift to maltose consumption. As *K. marxianus* strains cannot consume maltose,[30] only the *S. cerevisiae* part of coculture was able to produce CO_2 after depletion of glucose and fructose. Despite the small drop in CO_2 production after 2 h of fermentation, the single dosage coculture seems more suitable for bread making than the *K. marxianus* monoculture, as the productive fermentation time of the first was longer and because it reached higher CO_2 production rates.

The CO_2 production rate of the double dosage coculture was very high (≈1.7 mL/min) during the first hour of fermentation, indicating that all yeast cells (both Sc1 and Km1) in the culture were quickly consuming the glucose and fructose derived from fructan and sucrose hydrolysis. As the double dosage coculture contains more (fermenting) yeast cells, it is assumed that fructose and glucose, the primary fermentation substrates, will be depleted earlier in doughs fermented with this coculture, which is confirmed by the data represented in Figure 1B,C. The depletion of glucose and fructose was also reflected in a drop in CO_2 production after 60 min of fermentation. Around approximately 75 min of fermentation, the CO_2 production rate of the double dosage coculture reached similar rates as the Sc1 monoculture (≈1 mL/min). This might indicate that the *S. cerevisiae* part of the culture was consuming maltose and therefore produced CO_2, while the *K. marxianus* part of the culture was not fermenting anymore. The CO_2 production rate of the double dosage coculture remained around 1 mL/min until approximately 150 min of fermentation, after which it dropped, probably due to sugar depletion.

Summarizing, it can be concluded that Km1 produced relatively low amounts of CO_2 during fermentation, which makes it necessary to prepare cocultures of both Km1 and Sc1 to ensure sufficient production of CO_2 during the fermentation process. Despite the fact that the use of cocultures often results in domination of one of the two strains, it seems that the *S. cerevisiae* and the *K. marxianus* cells present in the cocultures

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were both producing CO_2 at relatively high rates when fructose and glucose were still present in the dough. On the basis of the CO_2 production profiles, it can be assumed that Km1 stopped contributing to the production of CO_2 when fructose and glucose were depleted. Finally, it can be concluded that the single dosage coculture produced similar levels of CO_2 as a monoculture of 100% Sc1 during the first 2 h of fermentation and therefore seems suitable for bread making.

Volume of Breads Prepared with Different Yeast Cultures. The volume of breads prepared with Km1 and the cocultures were compared with the volume of bread prepared with the commercially used *S. cerevisiae* strain Sc1 (Figure 3).

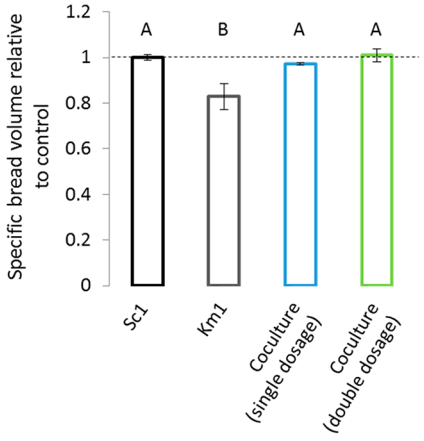

Figure 3. Specific bread volumes (relative to a control Terroir whole meal bread prepared with *Saccharomyces cerevisiae* bakery strain Sc1) of Terroir whole meal breads prepared with *Kluyveromyces marxianus* strain Km1, a single dosage coculture and a double dosage coculture. The single dosage coculture contains 1.06% yeast dry matter (on whole meal basis), the double dosage contains 2.12% yeast dry matter (on whole meal basis). Vertical bars represent standard deviations on measurements of biological triplicates. Means with different letters are significantly different ($P < 0.05$).

As expected based on the CO_2 production profiles, the volume of breads prepared with Sc1 and the cocultures was not significantly different. Indeed, the production of CO_2 by these cultures during proofing (90−126 min) was comparable and sufficient for optimal loaf volume. Breads prepared with the Km1 monoculture, however, had a significantly lower volume than breads prepared with Sc1, due to the low production of CO_2 by Km1 during proofing. The lack of maltose consumption by Km1 results in a lower production of CO_2 and a shorter productive fermentation time, leading to a lower bread volume. This problem might be solved by supplementation of dough with non-FODMAP sugars that can be fermented by *K. marxianus* like, for example, glucose. Another possible solution is the addition of enzymes that release glucose instead of maltose from damaged starch, like glucoamylase.[46,47]

Fructan Degrading Capacity of Different *Kluyveromyces marxianus* Strains. *K. marxianus* strain Km1 degraded the wheat grain fructans present in Terroir whole meal almost completely (>90%) during fermentation. To see if this also is the case for other strains, two other *K. marxianus* strains were tested. First, the CO_2 production rate of the two strains (Km2 and Km3) was tested in Terroir whole meal dough (Figure 4A). The strain codes of Km2 and Km3 were MUCL29917 and MUCL53775, respectively. Since the CO_2 production rate of both strains was very low (≤0.3 mL/min), cocultures of 5.3% *K. marxianus* (Km2 or Km3) and 5.3% *S. cerevisiae* Sc1 were used for dough and bread making trials. As with the previously used cocultures, the moisture content of the yeast pellets was approximately 80%, indicating that the amount of yeast dry matter added to dough was 2.1% on whole meal basis. The CO_2 production rate of the cocultures was comparable or slightly higher than that of Sc1, indicating that the production of CO_2 in the coculture was mainly attributed to the presence of the *S. cerevisiae* cells. As observed with Km1, cocultures of Sc1 with Km2 or Km3 degraded all the fructans present in Terroir whole meal dough after 2 h of fermentation (Figure 4B). This indicates that the combination of invertase and inulinase synthesized by these cocultures was sufficient to degrade the wheat grain fructans present in Terroir whole meal. As expected based on the CO_2 production profiles, bread volumes of breads prepared with cocultures of Sc1 with Km2 or Km3 were not significantly different from the volume of breads prepared with *S. cerevisiae* Sc1 alone (Figure 4C). Fructose and fructan levels present in breads prepared with the cocultures were ≤0.1% dm.

The results indicate that cocultures of *S. cerevisiae* and two other *K. marxianus* strains were able to fully degrade wheat grain fructans during bread making. The *K. marxianus* strains showed, however, only very low CO_2 production rates in dough when they were used as monoculture. Therefore, addition of *S. cerevisiae* cells was required for sufficient CO_2 production and optimal loaf volumes. This means that the *K. marxianus* part of the cocultures in this case mainly contributed to fructan degradation and not to dough leavening. Addition of (high dosages of) enzymes (inulinase) might give the same results.

Differences between *Kluyveromyces marxianus* inulinase and *Saccharomyces cerevisiae* invertase. *K. marxianus* inulinase degraded the fructans present in whole meal faster and to a greater extent than *S. cerevisiae* invertase, which might be explained by the different substrate specificity of both enzymes.[32] Sainz-Polo et al.[48] described that the structure of invertase plays a determinant role in its substrate specificity. The enzyme has an octameric structure, best described as a tetramer of dimers. The dimeric structure sets steric constraints that limit the access to the active site of oligosaccharides of more than four units.[48] Since the mean DP of wheat grain fructans is 4 to 5,[34] and the maximal DP was reported to be 19,[49] it is possible that a part of the fructans present in wheat do not fit properly in the active site of invertase. To verify this hypothesis, different substrates (inulin, mean DP ≥ 23; FOS, DP 2−8; and wheat grain fructans, mean DP 5.7) were incubated for 2 h with an overdose of pure invertase at 50 °C and pH 5, the optimal conditions for invertase (Table 2). As a control, the different substrates were also incubated with a fructanase mixture, containing endo- and exoinulinase, that is able to hydrolyze higher DP fructans such as inulin completely.

Quite surprisingly, the results indicated that invertase was able to fully degrade FOS with a DP of 2 to 8. HPAEC-PAD profiles of the FOS solution before and after incubation revealed that also the FOS with a DP > 4 were completely degraded by invertase (Figure S1 in the Supporting Information). When wheat grain fructans from whole meal were incubated with pure invertase, more than 90% of the fructans were degraded, which is a lot more than what was observed during dough fermentation. The part that was not degraded (8%) might have a DP that is too high for hydrolysis by invertase. As expected, invertase degraded inulin only partially (42% degradation), which can be explained by its

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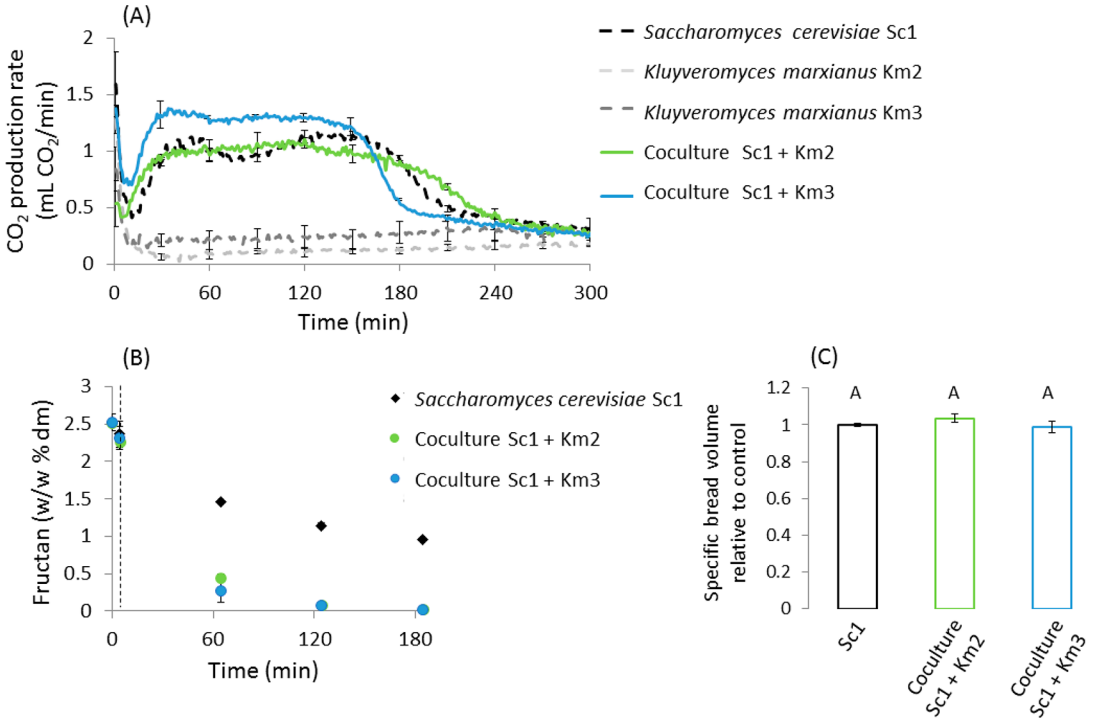

Figure 4. (A) CO_2 production rate (mL CO_2/min) of the *Saccharomyces cerevisiae* bakery strain Sc1, *Kluyveromyces marxianus* strains Km2 and Km3 (1.06% yeast dry matter on whole meal basis) and cocultures of Sc1 + Km2 (2 × 1.06% yeast dry matter) and Sc1 + Km3 (2 × 1.06% yeast dry matter) during Terroir whole meal dough fermentation (30 °C). (B) Fructan concentrations in dough samples fermented with *Saccharomyces cerevisiae* bakery strain Sc1 (black rhombus), coculture Sc1 + Km2 (green circle), and coculture Sc1 + Km3 (blue circle). Fructan concentrations were measured after 60, 120, and 180 min of fermentation and are expressed as weight percentages on whole meal dry matter base (w/w % dm). The first time point (t = 0) shows fructan concentrations in Terroir whole meal. The vertical dashed line indicates the time point after mixing. (C) Specific bread volumes (relative to a control Terroir whole meal bread prepared with *Saccharomyces cerevisiae* bakery strain Sc1) of Terroir whole meal breads prepared with coculture Sc1 + Km2 and coculture Sc1 + Km3. Vertical bars represent standard deviations on measurements of biological triplicates. Means with different letters are significantly different (P < 0.05).

Table 2. Degradation of Different Substrates (Fructo-Oligosaccharides (FOS), Inulin, Wheat Grain Fructans) with Invertase and Inulinase[a]

substrate	before incubation	after incubation with invertase	degradation by invertase (%)	after incubation with inulinase	degradation by inulinase (%)
FOS (DP 2−8) (mg/mL)	0.54 ± 0.01	0.00 ± 0.00	100	0.00 ± 0.00	100
inulin (DP ≥ 23) (mg/mL)	0.52 ± 0.01	0.30 ± 0.02	42	0.00 ± 0.01	100
wheat grain fructans (mean DP 5.7) (mg/mL)	0.51 ± 0.04	0.04 ± 0.02	92	0.02 ± 0.03	96

[a]FOS, inulin, and wheat grain fructan levels were measured before and after incubation with invertase and inulinase (2 h, pH 5, 50 °C), and the total degradation of substrate (%) was calculated. Experiments were performed in triplicate and standard deviations are shown in the table.

lower specificity toward higher DP fructans. Inulinase degraded inulin and FOS completely, and also 96% of the wheat grain fructans were degraded by inulinase (Table 2). These results confirm that inulinase has a higher specificity toward fructans with a high DP like high DP inulin, as reported.[32,50] Yet, also invertase can degrade oligosaccharides with a DP > 4. This was also observed by Nilsson et al.,[24] who analyzed the degradation of fructans with different DPs by yeast invertase during mixing and fermentation. About 50% of the trisaccharides, 40% of the tetrasaccharides, 20% of the pentasaccharides, and approximately 10% of the fructans with higher DP were degraded during mixing (10 min). Most of the remaining tri- and tetrasaccharides and 50% of the pentasaccharides were degraded during the first hour of fermentation, while 30% of the fructans with a higher DP were degraded during that time period.[24]

Additionally, the results also showed that pure invertase degraded more than 90% of extracted wheat grain fructans in a buffered solution, revealing that invertase is also able to degrade branched fructan structures, as wheat grains contain branched graminan- and neo-type fructans.[51] These observations might indicate that also other factors than only substrate specificity limit the degradation of wheat grain fructans by *S. cerevisiae* during bread dough fermentation.

Another reason for the lower activity of *S. cerevisiae* invertase toward wheat grain fructans during dough fermentation might be that invertase is mainly retained in the cell wall, while inulinase has both a cell wall associated and a secreted form.[33] It might be possible that fructans with a high DP cannot penetrate the cell wall and are therefore not accessible for invertase.[33] When an overdose of commercial *S. cerevisiae* block yeast (16% on whole meal basis) was added, however, 93% of the fructans present in Terroir whole meal were degraded

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during dough fermentation (2 h), indicating that at least 93% of the wheat grain fructans were able to penetrate the yeast cell wall. Moreover, this also suggests that addition of very high dosages of yeast (≥3 × 5.3%) results in almost complete fructan degradation. These high dosages would, however, result in excessive CO_2 production during proofing and collapse of the dough structure and might lead to off-flavors.

These observations suggest that the incomplete degradation of wheat grain fructans by *S. cerevisiae* invertase during dough fermentation is probably resulting from a combination of different factors, including (i) a lower specificity of invertase toward oligosaccharides with a high DP and (ii) a lower specific activity of invertase or (iii) a lower amount of enzymes synthesized by *S. cerevisiae* compared with *K. marxianus*. Further research is necessary to make a distinction between these factors.

Future Perspectives. In this study, a yeast-based strategy was developed to efficiently reduce FODMAP levels in dough and bread. It was shown that *K. marxianus* is more effective in degrading wheat grain fructans than *S. cerevisiae*. The *K. marxianus* yeast culture produced, however, only low amounts of CO_2 in lean dough because it lacks the ability to consume maltose. Therefore, cocultures of both *S. cerevisiae* and *K. marxianus* were prepared to ensure sufficient production of CO_2 during fermentation and hence optimal loaf volume. Loaf volume is, however, not the only determining factor for bread quality and therefore further research is necessary to reveal if other components produced by *K. marxianus* such as organic acids, glutathione, or aroma compounds negatively or positively affect dough rheology and/or bread flavor.

The major challenge for the production of low FODMAP breads with *K. marxianus* as leavening agent is to ensure sufficient production of CO_2 during fermentation. Next to preparing cocultures, as described in this study, other solutions such as the addition of carbon sources to dough that are (i) not a FODMAP and (ii) can be fermented by *K. marxianus* might be suitable to overcome the poor CO_2 production rate of *K. marxianus*. Examples of such carbon sources are glucose or sucrose, provided that the fructose generated by sucrose degradation is consumed by the yeast cells during the bread making process. Another possibility is to add enzymes that generate sugars that are fermentable by *K. marxianus*, like, for example, glucoamylase. Glucoamylase is known to cleave glucose from the nonreducing end of malto-oligosaccharides derived from starch, leading to the release of glucose during fermentation.[46] Therefore, this enzyme can be a suitable additive to increase the fermentation rate of *K. marxianus* strains.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02793.

HPAEC-PAD profile of a fructo-oligosaccharide solution before and after incubation with 100 U invertase (2 h, 50 °C, pH 5) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

FODMAPs, fermentable oligo-, di-, and monosaccharides and polyols; *S. cerevisiae*, *Saccharomyces cerevisiae*; *K. marxianus*, *Kluyveromyces marxianus*; dm, whole meal dry matter; OD, optical density; DP, degree of polymerization; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; FOS, fructo-oligosaccharides

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